

RESEARCH PAPER

Study Antibacterial Activity of Silver Nanoparticles Synthesis by Fenugreek Seed Extract against Resistant *Vibrio cholerae* Isolated from Owner of Birds

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ABSTRACT

The biosynthesis of AgNPs by using *Trigonella foenum-graecum* seed extract act as the reducing agents and have antibacterial activity. This research work was designed to evaluate the antibacterial effect of (AgNPs) and synergistic effect with antibiotic on resistant *V. cholerae* isolate. Synthesis of AgNPs by using fenugreek seeds and Characterization were diagnosed by UV-Visible spectroscopy, Fourier-transform infrared spectroscopy (FTIR), Scanning electron microscope (SEM) Transmission electron microscopy (TEM), X-ray diffraction and Evaluation of synergistic AgNPs in combination with antibiotic in well diffusion assay. The appearance of a dark brown colour in the solution indicates the presence of Ag NPs. The AgNPs characterized by UV peak appear at 450nm., TEM images showed nanoparticles spherical shape, size (10-50) nm, SEM showed size ranged between (26.87-47.93) nm. The FTIR with different distinct (7) peaks, in XRD the average was 17.86 nm. the synergistic of AgNPs with antibiotic in well assay showed that the antibiotic became more effective on *V. cholerae*. In Conclusion: AgNPs were effectively act as antibacterial against *V. cholerae* and using fenugreek seeds extract is considered better antibacterial regarded the low cost product and eco-friendly in AgNPs synthesis.

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INTRODUCTION

Cholera, an acute diarrheal disease caused primarily by consumption of water or food containing *V. cholerae* [1,2]. *V. cholerae* comma-shaped rod bacteria with polar flagella wrapped in a sheath, gram-negative and halophilic [3-5]. Nanoparticles a cluster of atoms having a large surface area with width little than 100 nm and are used in various applications including drug delivery. Antimicrobial lactivity, food industry

[6-8]. Silver nanoparticles (AgNPs) a substances capable of suppressing microorganism activity act as antimicrobial, anti-inflammatory antioxidant and anticancer [9-11]. Plant extracts act as reducing agents in AgNPs synthesis and capping agents Alternative types of plant extracts have been reported in the literature for green synthesis. [12-14]. Fenugreek seeds El Helba, are known in ancient before 2500 years ago is one of the important spices with various health

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benefits widely grown in Pakistan, India, Egypt, and Middle Eastern countries and it is one of the medical plants that has been reported for various pharmacological activities contain L-tryptophan, lysine-, mucilaginous fiber, saponins, coumarin, fenugreekine, nicotinic acid, phytic acid, scopoletin, and trigonelline. [15-17]. Other constituents are vitamins flavonoids, alkaloids, and amino acids, alkaloids fenugreek effects as anti-cancer drugs, hormones, antihypertensive, hypolipidemic, hypoglycemic, hyperinsulinemic, antidiuretic effects, antinociceptive, anti-inflammatory, properties. dyspepsia, bronchial asthma and antitussive other uses in cook as flavoring agents [18,19]. Extract of the fenugreek seeds act as antioxidant interaction between NPS and plant cells change biochemical reaction [20]. The main objective of current investigation is to focus on synthesize AgNPs by biological method using aqueous seeds extract of Fenugreek and study antibacterial effect.

MATERIALS AND METHODS

Preparation of Fenugreek seed extract

To prepare extract 20 g of seeds, grinding, boiled in 200 ml D.W /15 minutes, kept at room temperature for 24 hrs then filtration with filter paper. The seed extract was stored at 40 °C [21].

Synthesis of Silver Nitrate solution

Solution prepared by dissolving 8.49 g of AgNO_3 in 500 ml D.W, at room temperature for about 30 minutes under magnetization [21].

Synthesis of nanoparticles

Fenugreek extract 15 ml add to 135 ml solution of silver nitrate to make final volume 150 ml were kept in Shaking incubator for about 24 hrs at 30°C. change in color of the solution from pale yellow to reddish brown took place indicating the formation of silver nanoparticles. Silver nanoparticles solution was centrifuged at 15,000 rpm for 30 minutes. The pellets obtained washed by deionized sterile water for 3 times discarded supernatant. the pellets obtained were dried 65°C [22].

Characterization of biosynthesized AgNPs

UV-Vis spectroscopy analysis

Measurements the optical Absorbance spectra of the AgNPs were performed by suspending the nanoparticles in deionized water then sonicated

for 15 minutes transferred the suspension to a quartz cuvette cell. the visual observation of changing in color of fenugreek extract after treatment with silver nitrate mean detection of AgNPs by appearance of the dark brown which indicating the formation of silver nanoparticles due to reduction of pure silver ions, scanning the absorbance spectra in 400–700nm range of wavelength at a resolution of 1nm [23].

Fourier Transform Infrared Spectroscopy (FTIR)

The interaction between biomolecules and biosynthesized SNPs was analyzed using FTIR spectrophotometer in the range of 750-4000 cm^{-1} . Interaction responsible for capping, reduction, and stabilization of the SNPs. powder samples were mixed with the binding agent (KBr) and put into discs at high pressure, then discs were scanned to obtain the FTIR spectra [24].

Scanning Electron Microscope (SEM)

SEM used electron beam, with small probe by lenses restored across the nanomaterials. interactions with surface of sample, lead to emission of electrons penetration of nanoparticles into surface. These emitted electrons collected by a detector on monitor to give information about shapes and measure dimensions nanomaterial [25].

Transmission Electron Microscopy (TEM)

The shape, crystal structure and size of particles are shown by TEM. Analysis was done by put a drop in suspension of silver nanoparticles on a copper grid coated with carbon then water evaporate within a vacuum dryer [26].

X-ray diffraction (XRD) measurement

XRD was done by applying drop of nanoparticles suspension to the well plate dried on a glass substrate until the well plate's surface was covered in a uniform coating of particles. Reordering Diffraction signal intensity was done by using Diffract Plus. Scherer's, equation used for calculation of grains size from the width of the XRD planes [27].

Inoculum preparation of V. cholerae isolate

V. cholerae colonies was suspending in 5 ml of sterile normal saline shaking vigorously by using a vortex mixer for about 15 second. The turbidity of suspension was adjusted to 0.5 McFarland's

standards. These suspensions were further diluted to obtain a final concentration of inoculum (1.5×10^5 cell/ml [28].

Preparation of Stock Solution

A stock solution of silver nanoparticles and

Antibiotic stock solution was made by combining 10 milliliters of distilled water ($1000 \mu\text{g/ml}$) with 0.1 milligrams of AgNPs. Subsequently, distinct concentrations were created by combining distilled water with a specified volume from the stock solution. Antibiotic stock solution



Fig. 1. Culture bottles containing (A) AgNO_3 , (B) fenugreek seed extract without AgNO_3 and (c) Mixture of fenugreek seed extract with 1Mm AgNO_3 after incubation for 24hrs.

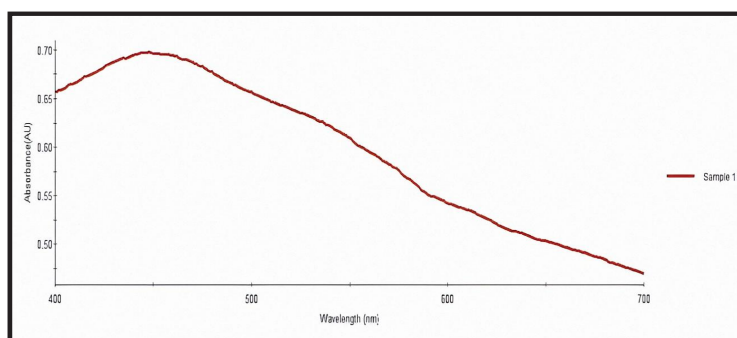


Fig. 2. UVVis spectrophotometer analysis of biologically synthesized silver nanoparticles of *Trigonell foenum-graecum* extract.

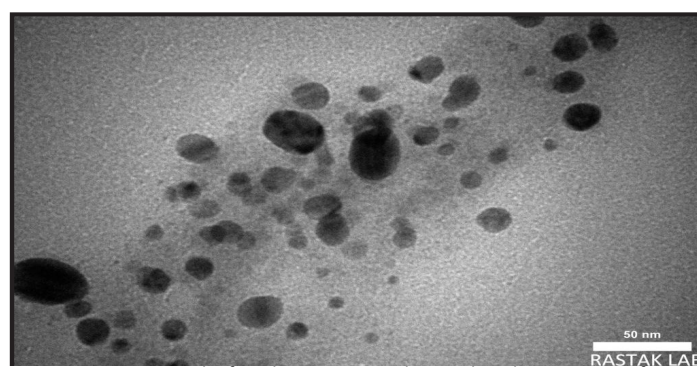


Fig. 3. TEM micrographs for silver nanoparticles produced, size ranging from 10 to 50 nm

was made by combining 0.1mg of (Gentamicin, Trimethoprim, Ceftazidime, Cefepime, Ampicillin, levofloxacin) with 10 ml of D.W (1000mg/ml). Then different Concentrations were created by combining distilled water with a known volume of the stocksolution [29,30].

Evaluation of synergistic potential of biosynthesized AgNPs in combination with antibacterial agents

In this study, the enhancement of antibacterial activity of antibiotics with AgNPs was investigated by using well diffusion method. 0.1 ml of *V.cholerae* strain was swabbed on the surface of Muller Hinton plates and after plate dried well of 5

mm created on surface agar by using of sterilized pasture pipette end. The wells were loaded with 100 μ L of freshly prepared AgNPs, 100 μ L of each antibiotic with a specific potency, and 100 μ L of each antibiotic in combination with AgNPs (50 μ L of antibiotic + 50 μ L of freshly prepared AgNPs solution). Wells contained D.W. and plant extract were considered as control. All these assays performed in triplicate. plates were incubated at 37 $^{\circ}$ C for 24hr. At the end of incubation time, the diameters of inhibition zone around the wells were measured and the mean value was expressed in millimeters. Criteria of sensitivity and resistance of antibacterial agents were recorded according

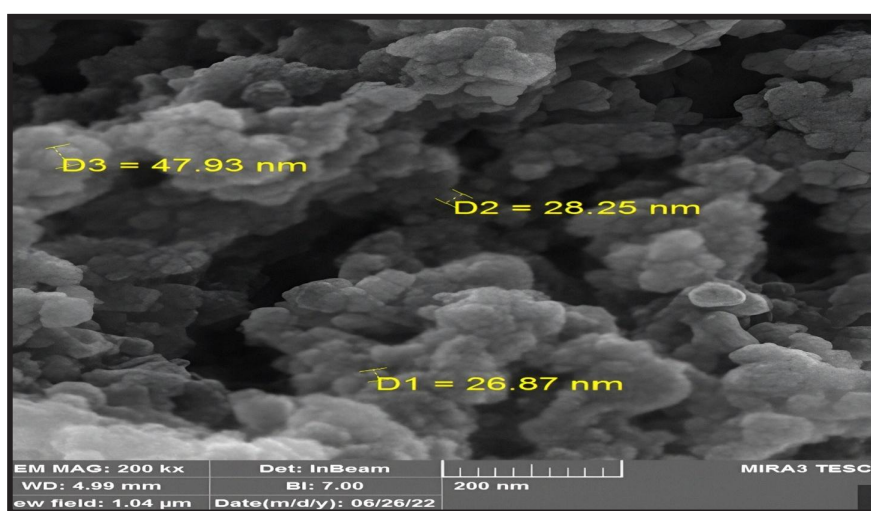


Fig. 4. SEM image of Ag NPs produced by *Trigonella foenum-graecum* seed extract with average size ranges between 26.87-47.93nm.

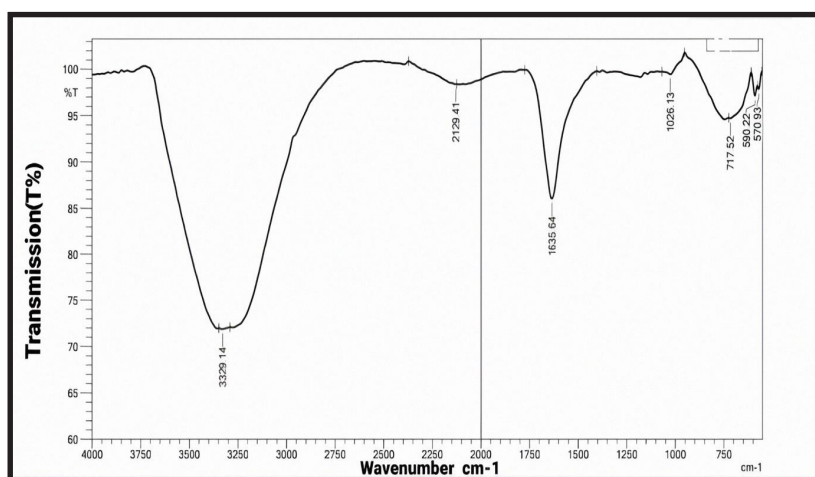


Fig. 5. FTIR spectra of silver nanoparticles synthesized using fenugreek seed extract

to [31]. Estimation of fold area was evaluated by calculating the inhibition zone produced by the antibacterial agent alone and in combination with biosynthesized SNPs [32].

Statistical Analysis

The Statistical analysis was detected the difference factors in study parameters (ANOVA) to significant compare between means [33].

RESULT AND DISCUSSION

Characterization of silver Nanoparticles

Silver nanoparticles were created when AgNO_3 solution and seed extract were combined, the

mixture's color initially changed from yellowish to brown after 1 hour. The color's intensity then increased with the prolonged period of incubation, and after 24 hours. when the reaction with Ag^+ ions was complete, the color changed to dark brown. The test color changed, which is a definite sign that AgNPs were forming in the reaction mixture as in (Fig. 1). AgNPs by UV characterized at prominent peak 450nm the range of AgNPs (Fig. 2). TEM images showed surface appearance and the morphological properties of AgNPs nanoparticles spherical shape, size (10-50) nm (Fig. 3). SEM characterized by showed spherical small size the average size of the nanoparticles ranged between

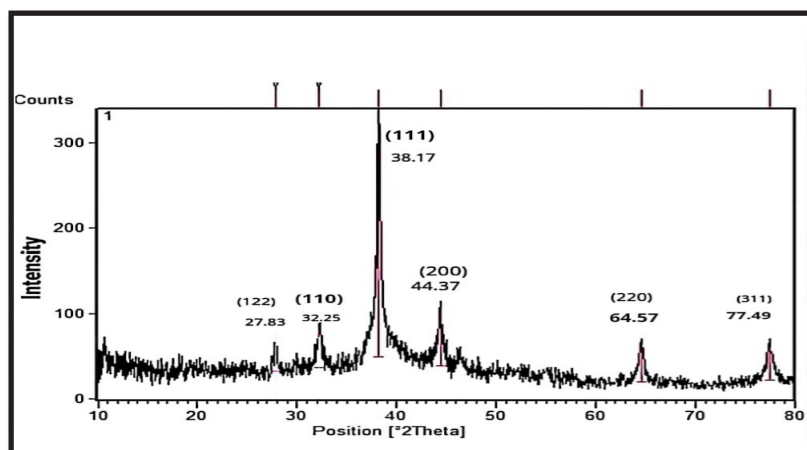


Fig. 6. XRD patterns of Silver Nanoparticles synthesized by fenugreek seed extract with AgNO_3 aqueous solutions.



Fig. 7. Antibacterial activity of AgNPs against *V.cholerae* : (A) AgNPs alone (B) (Control)by using deioniozed water.

26.87-47.93 nm (Fig. 4). The FTIR functional groups appear as peaks in the FTIR Spectra in the Fig. 5. In XRD the average silver nanoparticle particle size, which was found in this study to be 17.86 nm (Fig.6).

The present finding in UV-Vis spectrophotometer can be attributed due to excitation of free electrons this agree with many studies that reported the absorbance peak of biosynthesized AgNPs from *Trigonella foenum graecum* seed extract was observed at 450nm. [34,35]. This result of TEM and SEM agreed with results of [36-38]. In the FTIR functional groups Similar findings were found in

many studies that used seed extract as the source of silver nanoparticles [39,40].

Evaluation of synergistic potential of biosynthesized AgNPs in combination with antibacterial agents

The synergistic effect of synthesized AgNPs combined with 6 antibiotic was evaluated by measuring the diameter of the inhibitory zone around the conventional anti bacterial drugs against *V.cholerae* using standard well diffusion method. According to the results obtained by the susceptibility test of the standard antibacterial alone, *V.cholerae* was resistant to Ampicillin,



Fig. 8. (C) Antibacterial activity of *Trigonella Foenum Graecum* Seeds extract against *V.cholerae* (D) Antibacterial activity of deionized water and AgNO_3 .

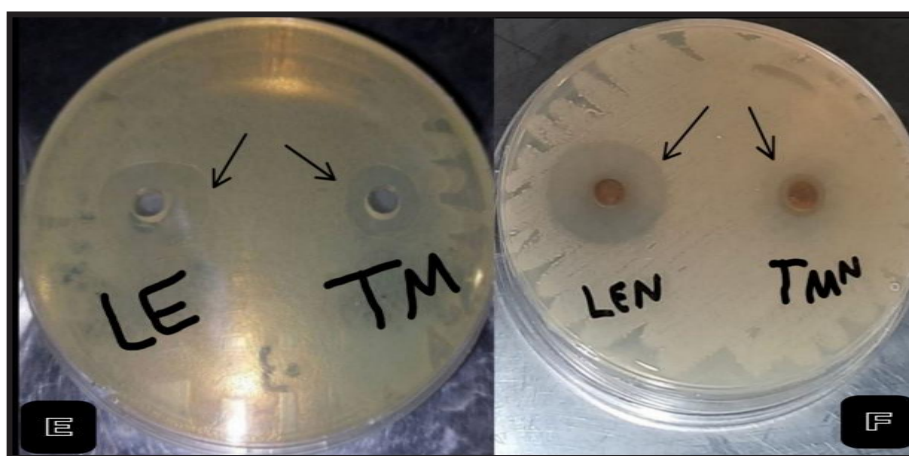


Fig. 9. (E) Antibacterial activity of Levofloxacin alone and Trimethoprim alone against *V.cholerae* (F) Antibacterial activity of AgNPs, against *V.cholerae* and their synergistic effect SNPs+ Levofloxacin (LEN) and SNPs + Trimethoprim (TMN).

Gentamicin, Ceftazidime, Cefepime, Trimethoprim, and susceptible to Levofloxacin. The biosynthesized AgNPs alone exerted significant activity against *V. cholerae* resulted in formation of varying zone of inhibition of *V. cholerae* which showed an inhibitory zone diameter of 18 mm. (Fig. 7). Inhibition zones were observed when they *Trigonella Foenum Graecum* Seeds extract was used and recorded 14 mm for tested *V. cholerae* and No inhibition zones were observed when deionized water was used, while the inhibitory zone diameter of 12mm was recorded in case of AgNO_3 as in Fig. 8. The synergistic activities of AgNPs in the presence

of antibiotic showed effectiveness against *V. cholerae* and mostly enhanced the antibacterial effect against *V. cholerae*. The inhibition zone of Levofloxacin alone 17 mm and when synergistic with AgNPs become 24mm and Trimethoprim alone 10mm with synergistic become 15mm as as in Fig. 9. Also, Ceftazidime alone (12 mm), with AgNPs become 16mm, Cefepime alone 14mm and with synergistic 20mm as in Fig. 10 while Ampicillin alone (12mm) with synergistic (20mm), Gentamicin alone 10mm with synergistic 15mm as in Fig. 11. The comparison between the inhibitory effects of antibiotic alone and combination with

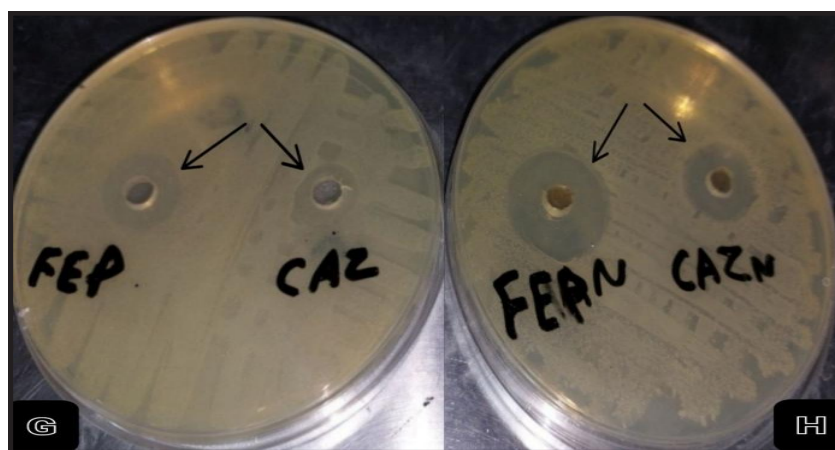


Fig. 10. (G) Antibacterial activity of cefepime alone and ceftazidime alone against *V. cholerae* (H) Antibacterial activity of AgNPs, against *V. cholerae* and their synergistic effect SNPs + Cefepime (FEPN) and SNPs + Cef tizidime (CAZN).

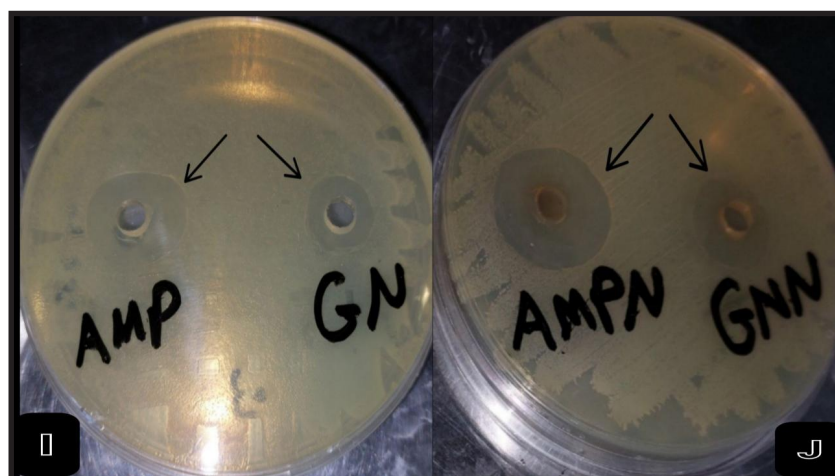


Fig. 11. (I) Antibacterial activity of Ampicillin alone and Gentamicin alone against *V. cholerae*. (J) Antibacterial activity of AgNPs, against *V. cholerae* and their synergistic effect SNPs + Ampicillin (AMPN) and SNPs + Gentamicin (GNN).

SNPs against *V.cholerae* as zone of inhibition as shown in Table 1.

The presence of *V.cholerae* in birds suggested a zoonosis important, Infection occurs while birds are feeding in areas that have been contaminated by humans or animals, and they may spread this pathogenic organism to other areas [41-45]. Research interests nanoparticles as antibacterial agents have a wide variety of harmful effects on microbes nanoparticles are more germicidal than silver metal in terms of mass. nanoparticles have a ability to adhere and penetrate bacterial membranes and accumulate inside cells, releasing silver ions continuously inside the cell [46-48]. In comparison to the standard antibacterial agents which are frequently used in the treatment of Vibriosis such as Ampicillin, Gentamicin, Ceftazidime, Cefepime, Trimethoprim and Levofloxacin, the AgNPs exhibited variable effects. The significant differences were demonstrated between AgNPs, Levofloxacin and plant extract, this implies that AgNPs have a strong antibacterial effect. Which was significantly higher than Levofloxacin and plant extract while this isolate of *V. Cholerae* was resistant to other antibacterial drug used in this study. Many studies proved the increase of resistance of *V.cholerae* isolates to antibiotics [49]. The antibacterial activity of AgNPs against *V.cholerae* was confirmed in some other studies but the reported values were different

from those obtained in the present study. Such differences could probably be explained by the differences in the *V.cholerae* isolates tested, or may be attributed to the differences in the NPs synthesis method and nature of NPs used such as size, shape and stabilizing agents as mentioned by [50,51]. In study of [52] the inhibition diameter of AgNO₃ against *V.cholerae* was recorded at 17mm and this result agree with the present study. The low concentrations of Ag induce leakage in *Vibrio cholerae* membrane, in complete deenergization and, cell death as mentioned by [53]. Also in this study found synthesized silver nanoparticle from seed extract had antibacterial effect against *V.cholerae* and these results agreement with previous study that recorded inhibition diameter of seed extract of *Trigonella foenum-graecum* on *Streptococcus pneumoniae* (15 mm) *Proteus vulgaris* 10mm, *pseudomonas aeruginosa* 14mm, *Staphylococcus aureus* 9 mm while diameter of seed extract of *Trigonella foenum-graecum* SNP 17mm, 12mm, 18mm, 11 mm respectively in study of [54]. *Trigonella foenum-graecum* (fenugreek seeds) Cu nanoparticles was reached 5 mm for *Pseudomonas aeruginosa*, 10mm zone of inhibition for *Bacillus subtilis* 10mm for *Agrobacterium tumefaciens* 9 mm for *Proteus mirabilis* and while in study of [37].

The Silver nanoparticles (AgNPs) were synthesized using aqueous leaf extract of

Table 1. Show different susceptibility of *V.cholerae* to antibiotic alone and antibiotic synergistic with SNPs in Diameter of inhibitory zone.

Antibacterial	Diameter of inhibitory zone mm)	Antibacterial with SNPs	Diameter of inhibitory zone mm with SNPs
Silvernanoparticle (SNPs)	18	-----	-----
Levofloxacin	17	SNPs + Levofloxacin	24
Trimethoprim	10	SNPs +Trimethoprim	15
cefepime	14	SNPs +Cefepime	20
ceftazidime	12	SNPs +Ceftazidime	16
Ampicillin	12	SNPs +Ampicillin	20
Gentamicin	10	SNPs + Gentamicin	15
P-value	4.027 *	P-value	5.178 *
* (P≤0.05).			

Trigonella foenumgraecum and found the zone of inhibition was recorded 9 mm for *Escherichia coli* O157:H7 and *Staphylococcus aureus*. Also, [38] performed effect of silver nanoparticles from water extract of *Trigonella foenumgraecum* and found the Zone of inhibition of AgNP for *S. aureus* was 18mm, *B. subtilis* 22mm, MRSA 14mm, *E. coli* 8mm, *P. aeruginosa* 12mm and *K. pneumoniae* 25 mm. On the other hand, the antibacterial activity of synthesized AgNPs leaf extract of *Trigonella foenumgraecum* was then tested on *S. aureus*, *E. coli*, *P. aeruginosa* and *V. cholerae* which showed an inhibition zone of 6, 12, 11 mm respectively as documented by [39]. The antimicrobial effect can be due to disruption in bacterial cell wall, generation of ROS, and release of Ag⁺ [55]. Therefore, their synergistic activity with different antimicrobials may be due more mechanisms. Gram-negative bacteria are with an outer membrane of lipopolysaccharide (LPS), which act as a layer limits penetration of antimicrobials with large molecular weights [56]. so AgNPs due to its reduced size, entered inside the cells of bacteria and effect on respiration process or by triggering cell membrane damage [57]. According to the previous studies the presence of different compounds in seeds of plant act as the reducing agent and form the stabilized AgNPs [58]. Most microorganism have shown silver ions to be highly toxic., Interference in DNA replication and inactivating proteins. Leading to bacterial death [59]. AgNPs and antibiotics combinations limit antibiotic resistance and dose requirements for treatment [60]. The bactericidal effect of silver nanoparticles was determined (*V.cholerae* -Levo) Levofloxacin have greater antibacterial activity. And enhanced antibacterial activity of AgNPs- Levo [61]. Combining nanometrial with antimicrobials minimize toxicity and reducing the requirement for high-dosage regimens [62]. studies found that AgNP- combinations make pathogens susceptible to ampicillin and ciprofloxacin, with significant changes in antibacterial activities and structural properties [63,64].

CONCLUSION

The purpose of this work was to evaluate the antibacterial impact of fenugreek seed extract-synthesised AgNPs. AgNPs were found to be efficient against *V. cholerae*, and fenugreek seed extract is thought to be a more potent antibacterial with a low cost of production that is

also environmentally to AgNP synthesis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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